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PRINCIPAL INVESTIGATOR: Martin A. Steffen, M.D., Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, MA 02118

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Martin A. Steffen, M.D., Ph.D.; Agnes Bergerat

Email: steffen@bu.edu

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14. ABSTRACT

During this third year of research, we have analyzed protein abundance levels of CD4+ T-lymphocytes and demonstrated that in accordance with our original hypothesis, we are able to discriminate between prostate cancer and benign prostate disease in patients with an elevated PSA level. A manuscript detailing these results is being prepared. Additionally, we explored the proteomes of lymphocytes from mice, and have found that the procedures involved with sample procurement are less robust than for the human samples, encouraging us to focus on the human samples. We remain optimistic that we could potentially lay the groundwork for a test that would significantly reduce the number of prostate biopsies performed annually.

15. SUBJECT TERMS
Prostate cancer, biomarker, disease classifier, proteomics, leukocyte, mass spectrometry

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INTRODUCTION: This research has the long-term goal of developing a test that will discriminate between prostate cancer (PrCA) and benign prostate disease. The PSA test is sensitive for prostate cancer but not specific – many elevated levels of PSA are associated with the diseases of chronic prostatitis (CP) or benign prostatic hyperplasia (BPH) [1]. If, after an elevated PSA test, our test could distinguish among these possibilities, we could better take advantage of the sensitivity of the PSA test, with the enhanced specificity resulting from our test. Among other benefits, this would be significant for reducing the need for prostate biopsies, with all its attendant costs and potential morbidities [2,3,4]. Our approach is to look for biomarkers present on circulating leukocytes that will distinguish among the three conditions. We hypothesize that it is likely such differences exist because of the very different natures of the inflammatory response in those conditions [5,6]. The practical rationale for this approach is that circulating leukocytes are present in the easily accessible clinical specimen of blood, yet avoids the pitfalls of serum proteomics, in which highly abundant proteins make the identification of biomarkers extremely challenging [7]. More broadly, our proposed studies will add to our understanding of the complex relationship among inflammation, the immune system and the development of prostate cancer. This knowledge may aid in the earlier identification of cases destined to become clinically significant cancers, as opposed to those which may be present are not destined to achieve clinical significance [8].

BODY: We hypothesize that benign and malignant prostatic disease will each produce proteomic changes in circulating leukocytes characteristic of their disease. By analyzing the proteomes of leukocytes, we are now able to discriminate between the two classes of disease non-invasively. To achieve these objectives, we utilized mass spectrometry to produce comprehensive proteomic profiles of peripheral leukocytes from individuals with elevated PSA, and correlated the results with those obtained by histologic examination of core-biopsy samples. Supporting data towards these goals are detailed below.

After completion of patient recruitment, we selected seven control samples and seven cancer samples of CD4+ T-lymphocytes that were judged to be “high-quality samples” based on purity assessed by FACS analysis, and in amount of cells (Table 1). The two groups were matched in age, and PSA level. Proteins from the lymphocytes were digested with trypsin, and the resulting triptic peptides were purified using solid phase extraction columns.

The proteins were analyzed using a Thermo Scientific Orbitrap mass spectrometer. Each sample was analyzed twice, so the 14 samples produced 28 data sets. Proteins were identified using the MASCOT search engine (Matrix Science), and label free quantitation was performed using the peak ion intensities of the Progenesis software package (Nonlinear Dynamics). In total, we identified 1250 proteins, and obtained quantitative data for 922 of them. 385 proteins were identified as differentially expressed, with Anova p-values < 0.1.

	Sample#	Age	PSA level Prior Biopsy	Ethnicity	# of CD4 (in Million)	Purity	Biopsy results
CONTROL	P#14	63	6.1	Hispanic	7	96.5%	Biopsy was negative
	P#17	61	5.3	Jamaican	4.5	97.2%	Biopsy is negative and is post prosate cancer/cryotherapy
	P#20	70	13.6	African American	5	98.1%	TURP
	P#22	70	6.6	Indian	6	99.2%	chronic inflamation /minimal urinary symptoms
	P#26	53	5.22	Brazilian	12	98.0%	Inflammation
	P#31	69	10.4	Caucasian	6	99.1%	Inflammation acute and chronic -2006 -identical-
	P#34	57	5.7	Caucasian	14	99.0%	enlarged, inflammation PIN4
AVGS		63.3	7.56			98.1%	
		6.8	3.2			0.0	
		10.7%	42.4%			1.1%	
CANCER	P#12	59	3.2	Hispanic	22	98.7%	Gleason score of 10 in 3 cores
	P#15	60	4.4	Hispanic	9	97.8%	5 cores Gleason score was 10
	P#19	59	5.4	Black (Haitian)	9	97.4%	Gleason score 6
	P#24	57	13	Black (Jamaican)	16	98.5%	Adenocarcinoma - Gleason 6
	P#36	64	6	African American	6	98.4%	Gleason 6 + inflammation
	P#37	63	11.6	Caucasian	11	98.7%	Gleason 6 - 7
	P#39	62	7.5	African American	18	98.6%	Gleason 6
AVGS		60.6	7.3			98.3%	
		2.5	3.7			0.0	
		4.1%	50.5%			0.5%	

Table 1. Characteristics of patients from which samples were chosen for evaluation.

As we've discussed in detail previously [9], proteins can be differentially expressed, without being useful for class discrimination. We therefore selected a preliminary group of 65 proteins that appeared to discriminate between the two groups (Table 2). (We will refine the selection further using more sophisticated analytical approaches during our no-cost extension.)

Finally, we developed a simple signed-sum scoring metric, based on our previous efforts [9]. For each protein abundance measurement, a threshold value was determined, and a sample was given a positive score contribution if its level was above or below the threshold value. The total score from all 65 proteins are then computed for each sample, and plotted in figure 1. As can be seen, using this simple and preliminary approach, we can achieve reasonable separation between individuals with prostate cancer and benign prostate disease, even though each individual has an elevated PSA level. Two patients with pathology reports showing only enlargement or inflammation but no cancer had high scores. Because the samples were analyzed twice, there are four data points above the dashed line.

Accession	Description	Ratio CA/CTL
NP_079002.3	IQ and AAA domain-containing protein 1	0.189
NP_003082.1	small nuclear ribonucleoprotein-associated proteins B and B' isoform B	0.440
NP_000607.1	T-cell surface glycoprotein CD4 isoform 1 precursor	0.709
NP_003968.2	AH receptor-interacting protein	0.771
NP_149073.1	SAP domain-containing ribonucleoprotein	0.790
NP_001001670.1	protein FAM75D1	0.816
NP_002127.1	heterogeneous nuclear ribonucleoprotein A1 isoform a	0.864
NP_009035.3	60S ribosomal protein L10a	0.878
NP_005347.3	tyrosine-protein kinase Lck precursor	0.879
NP_004915.2	alpha-actinin-4	0.885
NP_958786.1	plectin isoform 1a	0.896
NP_002061.1	guanine nucleotide-binding protein G(i) subunit alpha-2 isoform 1	0.918
NP_009330.1	signal transducer and activator of transcription 1-alpha/beta isoform alpha	0.921
NP_036558.3	splicing factor 3B subunit 3	0.922
NP_005959.2	heterogeneous nuclear ribonucleoprotein M isoform a	0.923
NP_000961.2	60S ribosomal protein L6	0.951
NP_006079.1	tubulin beta-4B chain	0.956
NP_005817.1	heterogeneous nuclear ribonucleoprotein R isoform 2	0.963
NP_003133.1	lupus La protein	1.084
NP_003325.2	ubiquitin-like modifier-activating enzyme 1	1.092
NP_002289.2	plastin-2	1.094
NP_001419.1	alpha-enolase isoform 1	1.117
NP_001186040.1	malate dehydrogenase, cytoplasmic isoform 1	1.126
NP_001243650.1	T-complex protein 1 subunit delta isoform b	1.127
NP_001185771.1	T-complex protein 1 subunit beta isoform 2	1.136
NP_001152759.1	triosephosphate isomerase isoform 2	1.153
NP_055040.2	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	1.153
NP_005317.2	hydroxyacylglutathione hydrolase, mitochondrial isoform 1 precursor	1.163
NP_002782.1	proteasome subunit alpha type-6	1.169
NP_006139.1	LIM and SH3 domain protein 1	1.185
NP_001011.1	40S ribosomal protein S16	1.189
NP_003399.1	zinc finger protein 37 homolog	1.189
NP_002291.1	L-lactate dehydrogenase B chain	1.198
NP_001035918.1	serine/threonine-protein kinase MST4 isoform 2	1.202
NP_821068.1	serine/threonine-protein phosphatase 2A activator isoform a	1.206
NP_002779.1	proteasome subunit alpha type-3 isoform 1	1.212
NP_005331.1	histidine triad nucleotide-binding protein 1	1.216
NP_002787.2	proteasome subunit beta type-4	1.216
NP_066953.1	peptidyl-prolyl cis-trans isomerase A	1.221
NP_004559.4	serpin B6	1.221
NP_001159757.1	T-complex protein 1 subunit eta isoform d	1.245
NP_004896.1	peroxiredoxin-6	1.271
NP_000662.3	alcohol dehydrogenase class-3	1.273
NP_000185.1	hypoxanthine-guanine phosphoribosyltransferase	1.276
NP_002792.1	proteasome subunit beta type-10 precursor	1.277
NP_066997.3	DBIRD complex subunit KIAA1967	1.283
NP_001619.1	aldose reductase	1.288
NP_006704.3	activated RNA polymerase II transcriptional coactivator p15	1.289
NP_001254479.1	titin isoform IC	1.289
NP_000678.1	adenosylhomocysteinase isoform 1	1.302
NP_510880.2	unconventional myosin-XVIIIa isoform a	1.303
NP_000687.3	4-trimethylaminobutyraldehyde dehydrogenase	1.305
NP_009193.2	protein DJ-1	1.345
NP_068823.2	ubiquitin-conjugating enzyme E2 variant 1 isoform a	1.373
NP_002558.1	phosphatidylethanolamine-binding protein 1 preproprotein	1.377
NP_038477.2	bromodomain adjacent to zinc finger domain protein 2A	1.383
NP_004108.1	peptidyl-prolyl cis-trans isomerase FKBP5 isoform 1	1.407
NP_005611.1	protein S100-A11	1.411
NP_001005339.1	regulator of G-protein signaling 10 isoform a	1.467
NP_001035548.1	endoplasmic reticulum aminopeptidase 1 isoform b precursor	1.496
NP_001880.2	quinone oxidoreductase isoform a	1.553
NP_055955.1	tubulin--tyrosine ligase-like protein 12	1.566
NP_005309.1	histone H1.0	1.590
NP_057370.1	L-xylulose reductase isoform 1	1.675
NP_001230824.1	tumor protein D54 isoform j	2.317

Table 2. The 65 proteins used to build preliminary discriminator.

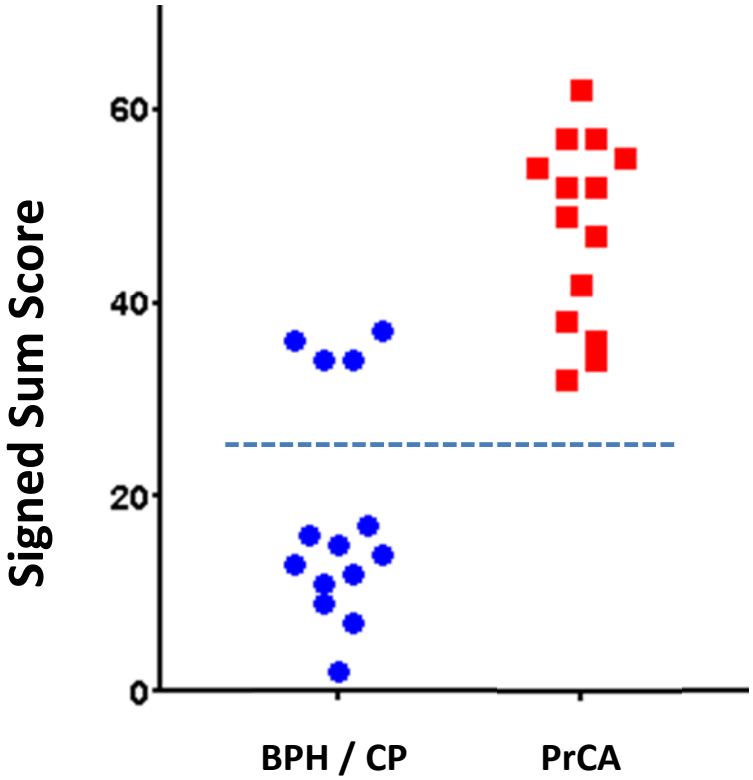

Figure 1. Discrimination between prostate cancer and benign prostate disease using protein abundance levels of 65 proteins from CD4+ T-lymphocytes. Blue circles - patients with prostate inflammation or enlargement. Red squares - patients with prostate cancer.

We also explored the proteomic investigation of leukocytes from mice in order to compare lymphocytes from two strains of mice that are essentially genetically identical but differ markedly in their propensity to develop prostate tumors driven by a transgene. The isolation of lymphocytes from one or more groups of mice was based upon a protocol developed by the alliance for cellular signaling (AfCS - AfCS Procedure Protocol PP0000000100, Version 1, 02/19/02), with slight modification. Lymphocytes from mouse spleens were isolated using negative selection with the appropriate monoclonal antibodies coupled to magnetic microbeads. This strategy depletes non-lymphocytic cells from a mixed population of splenocytes. Anti-Mac- 1/CD11b microbeads are included in the negative selection to improve the removal of myeloid cells. Lymphocye isolation was performed using an AutoMACS automatic magnetic bead cell sorter (Miltenyi Biotec). Results using tissue from 34 mice euthanized according to an IACUC-approved protocol are summarized in Table 3. In some cases, cells from multiple mice were pooled (*e.g.* mice 12 -1 6) in order to isolate larger number of cells. We found results to be inconsistent, with variability in cell purity and viability. For these reasons, along with the fact that our primary goal is the analysis of human cells, work that was proceeding acceptably, and the short amount of time available, we chose to not pursue this work further.

Mouse	# PBMCs after RBC lysis		Cell Lysis after transfer	PBMCs used for CD4selection		Lymphocytes recovered		
	cells (million)	% alive		cells (million)	% alive	cells (million)	% alive	Purity
1	112.5	73.3%	not visible	112	73.3%	7.6	98%	85.5%
2	112.5	56.0%	not visible	112	56.0%	7.6	95%	67.0%
3	129	52.3%	Major	62	90.07%	10	95%	91.0%
4	162	30.6%						
5	300	78.7%	Little lysis	290	86%	54	94%	
6	230	52.2%		180	84%	26	92%	
7	some RBCs left		Major	190	71%	16	88%	
8	80	40%	Little lysis	250	43%	15	89%	66%
9	104	30%	Little lysis					
10	88	36%	Little lysis					
11	114	40%	Little lysis					
12	290	15%	Little lysis	220	90%	20	90%	81%
13								
14								
15								
16								
17	210	86%	Little lysis	140	92%	17	90%	89%
18								
19								
20								
21			no visible lysis	60	83%	6	93%	84 -91%
22			Little lysis	210	83%	24	86%	88%
23								
24								
25	Positive Selection - Did not give any good results							
26								
27	20	Ficoll test						
28								
29	112	75%	Little lysis	75	87%	10	97%	90%
30								
31	20	Ficoll led to many lost cells - the centri need to be optimized - Stored as PBMCs						
32								
33	68		Little lysis	45	90%	5	90%	>89.7%
34	67		Little lysis	42		3.7	84%	>89.2%

Table 3. Summary of results of lymphocyte isolation from mice spleens.

KEY RESEARCH ACCOMPLISHMENTS:

- Have analyzed clinical sample proteomes from monocytes using shotgun mass spectrometry.
- Have demonstrated that the original hypothesis - that proteomic signatures of CD4+ lymphocytes has the potential to discriminate between prostate cancer and other prostate disease in the setting of elevated PSA levels.

REPORTABLE OUTCOMES:

1.) A manuscript detailing these results, which represent a significant step towards our long-range goal of developing a blood test that can discriminate between prostate cancer and benign prostatic disease is in preparation.

CONCLUSION: We remain enthusiastic about the prospects for the development of a test utilizing molecular differences in circulating leukocytes for the identification of patients with prostate cancer. Such a test would be clinically useful for reducing the number of biopsies performed, considerably reducing health care-wide expenditures associated with this procedure. The proteins we have utilized in our preliminary discrimination signature can be validated with mass spectrometry and antibody-based methods, allowing for high confidence in the observed differences. If the differences prove to be robust, it will be appropriate to evaluate the observations in wider clinical populations, ideally such as the Prostate Cancer Clinical Trials Consortium (PCCTC). If one can increase the certainty that an elevated PSA is a result of prostate cancer, and not due to nonmalignant prostate disease, one could pursue evaluating lower PSA levels as indicators of cancer, to increase the sensitivity of the test. This of course is documented in the literature, but it is always concerns about the higher false positive rates that lower levels of PSA are not considered suitable for routine clinical use.

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APPENDICES: N/A